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Islet distribution of Peptide YY and its regulatory role in primary mouse islets and immortalised rodent and human beta-cell function and survival

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Abstract

Recent evidence suggests that the classic gut peptide, Peptide YY (PYY), could play a fundamental role in endocrine pancreatic function. In the present study expression of PYY and its NPY receptors on mouse islets and immortalised rodent and human beta-cells was examined together with the effects of both major circulating forms of PYY, namely PYY(1-36) and PYY(3-36), on beta-cell function, murine islet adaptations to insulin deficiency/resistance, as well as direct effects on cultured beta-cell proliferation and apoptosis. *In vivo* administration of PYY(3-36), but not PYY(1-36), markedly ($p < 0.05$) decreased food intake in overnight fasted mice. Neither form of PYY affected glucose disposal or insulin secretion following an i.p. glucose challenge. However, *in vitro*, PYY(1-36) and PYY(3-36) inhibited ($p < 0.05$ to $p < 0.001$) glucose, alanine and GLP-1 stimulated insulin secretion from immortalised rodent and human beta-cells, as well as isolated mouse islets, by impeding alterations in membrane potential, $[Ca^{2+}]_i$ and elevations of cAMP. Mice treated with multiple low dose streptozotocin presented with severe ($p < 0.01$) loss of beta-cell mass accompanied by notable increases ($p < 0.001$) in alpha and PP cell numbers. In contrast, hydrocortisone-induced insulin resistance increased islet number ($p < 0.01$) and beta-cell mass ($p < 0.001$). PYY expression was consistently observed in alpha-, PP- and delta-, but not beta-cells. Streptozotocin decreased islet PYY co-localisation with PP ($p < 0.05$) and somatostatin ($p < 0.001$), whilst hydrocortisone increased PYY co-localisation with glucagon ($p < 0.05$) in mice. More detailed *in vitro* investigations revealed that both forms of PYY augmented ($p < 0.05$ to $p < 0.01$) immortalised human and rodent beta-cell proliferation and protected against streptozotocin-induced cytotoxicity, to a similar or superior extent as the well characterised beta-cell proliferative and anti-apoptotic agent GLP-1. Taken together, these data highlight the significance and potential offered by modulation of pancreatic islet NPY receptor signalling pathways for preservation of beta-cell mass in diabetes.

1. Introduction

Cells of the pancreatic islets of Langerhans have a vital endocrine function related to secretion of hormones that regulate of glucose homeostasis and nutrient metabolism. The physiological significance of the classical islet peptide hormones including insulin, glucagon and somatostatin is already well established (Unger et al., 1978). More recently, the existence of other non-classical islet regulatory peptide hormones has been described (Heller and Aponte 1995, Myrsen-Axcrona et al., 1997; Masur et al., 2005; Fujita et al., 2010; Hansen et al., 2011; Whalley et al., 2011, Marchetti et al., 2012). As such, ghrelin is now considered as a pancreatic islet hormone (Wierup et al., 2014), with an accepted function of regulating insulin secretion and blood glucose levels (Yada et al., 2014). In addition, the incretin peptides, namely glucagon like peptide-1 (GLP-1) and glucose-dependent insulintropic polypeptide (GIP), secreted locally from islet alpha-cells, have been shown to modulate beta-cell mass under situations of cellular stress (Moffett et al., 2014; Vasu et al., 2014; Moffett et al., 2015). Likewise, there is now emerging evidence to suggest that islet cell secretion and action of the gut hormone peptide YY (PYY), may be involved in the preservation of beta-cell mass and control of insulin secretion (Sam et al., 2012; Persaud and Bewick, 2014; Shi et al., 2015). In keeping with this, receptors that mediate the biological actions of PYY, namely neuropeptide Y (NPY) receptors, are thought to be expressed on pancreatic endocrine cells (Amisten et al., 2013).

PYY is a hormone that was first isolated from porcine gut in 1980, and shown to be closely related to pancreatic polypeptide (PP) (Tatemoto and Mutt, 1980). During embryonic development, the first islet cell type to appear is known to co-express glucagon and the PP family of peptides, including PYY (Mulder et al., 1998). This could suggest that PYY plays some part in beta-cell development, adaptation and ultimately insulin secretory function (Mulder et al., 1998). In agreement, selective destruction of PYY expressing cells in adult mice induces dysfunction and loss of beta-cells (Sam et al., 2012), whilst transgenic mice

overexpressing PYY have a tendency for increased insulin concentrations (Shi et al., 2012). Moreover, recent characterisation of transgenic mice selectively overexpressing PYY in pancreatic beta-cells revealed increased beta-cell mass and enhanced basal, but not glucose-stimulated insulin release (Shi et al., 2015). Interestingly, PYY knockout (*PYY*^{-/-}) mice exhibit hyperinsulinaemia (Boey et al., 2006), perhaps reflecting adaptive changes as a result of lifelong PYY deficiency. Taken together, it is apparent that modulation of PYY islet signalling could represent a potential new therapeutic approach to diabetes, which is typically characterised by a significant loss of beta-cell mass and function over time (Thorens, 2013).

There are two well characterised circulating forms of PYY, PYY(1-36) and PYY(3-36) (Grandt et al., 1994). PYY(3-36), the main circulating form generated by DPP-4 action (Medeiros and Turner, 1994), is largely recognised as the major bioactive PYY peptide responsible for the well documented anorectic actions, including inhibition of food intake, reduced gastric motility and reduction in body weight gain (Greeley et al., 1988; Pittner et al., 2004; Boey et al., 2007). However, at the pancreatic level PYY(1-36) has been shown in some studies to modulate insulin secretion from isolated rodent islets (Sam et al., 2012; Chandarana et al., 2013). Moreover, potential beneficial effects of PYY on pancreatic beta-cell survival may be linked to activation of NPY1 receptors, to which PYY(1-36) binds more strongly than PYY(3-36) (Sam et al., 2012). Therefore, in the present study we evaluated the effects of PYY(1-36) and PYY(3-36) on modulation of insulin secretion *in vivo* and *in vitro* from pancreatic clonal rodent BRIN BD11 and human 1.1B4 beta-cells, as well as mouse isolated islets. Furthermore, we assessed NPY receptor subtypes and potential mechanisms of PYY action on beta-cell insulin secretory function together with effects on beta-cell proliferation and apoptosis *in vitro*. Finally, we investigated whether changes in islet PYY expression are linked to the classical pancreatic architectural adaptations induced by streptozotocin and hydrocortisone treatment in mice. The results support the concept that PYY produced locally

within the islets may play an important role in the regulation of insulin secretion and preservation of beta-cell mass.

2. Materials and methods

2.1. Real time Reverse Transcription PCR

To determine pancreatic expression of PYY and its receptors, namely NPY1, NPY2, NPY4 and NPY5, mRNA was extracted from isolated mouse islets, clonal rodent BRIN BD11 cells and human 1.1B4 pseudoislets (Green et al., 2015) using an RNeasy Mini Kit following manufacturer's instructions (Qiagen, UK). mRNA (3 µg) was converted to cDNA using SuperScript II Reverse Transcriptase kit (Invitrogen, Paisley, UK). The reaction mix consisted of Quantifast SYBR green master mix (Qiagen, UK), primers (forward and reverse), cDNA and RNase free water. Amplification conditions were set at 95°C for initial and final denaturation, 58°C for primer annealing and 72°C for extension for 40 cycles, followed by a melting curve analysis, with temperature range set at 60°C to 90°C. Data were analysed using $\Delta\Delta C_t$ method and normalised to *Actb/ACTB* expression.

2.2 Animals

All animal studies were carried out using adult male C57BL/6 or NIH Swiss mice (12 weeks of age, Harlan Ltd, UK), housed individually in air conditioned room at 22±2°C with 12 hours light and dark cycle and *ad libitum* access to standard rodent diet (10% fat, 30% protein and 60% carbohydrate; Trouw Nutrition, Northwich, UK) and drinking water. All *in vivo* experiments were carried out in accordance with the UK Animal Scientific Procedures Act 1986.

2.3 Acute *in vivo* effects

Plasma glucose and insulin responses were evaluated after intraperitoneal (i.p.) injection of glucose alone (18 mmol/kg body weight) or in combination with test peptides (25 nmol/kg body weight) in overnight (18 h) fasted NIH Swiss mice. In a second series of experiments, 18 h fasted normal mice were used to assess the effects of respective test peptides on food intake. Mice received an i.p. injection of saline alone (0.9 % (w/v) NaCl) or in combination with test peptides (25 nmol/kg body weight) and food intake measured at 30 min intervals.

2.4 *In vitro* insulin secretion

Effects of peptides on *in vitro* insulin secretion were examined using immortalised rodent BRIN-BD11 and human 1.1B4 beta-cells, whose characteristics have been reported previously (McClenaghan et al., 1996; McCluskey et al., 2011). Importantly, BRIN-BD11 cells mimic the glucose sensitivity and overall secretory performance of other immortal beta-cell lines and represent a useful cellular model for studies of beta-cell function (Hamid et al., 2002). Cells were seeded (150,000/well) into 24-well plates (Nunc, Roskilde, Denmark) and allowed to attach overnight at 37°C. Following 40 min pre-incubation (1.1 mmol/L glucose; 37°C), cells were incubated (20 min; 37°C) in the presence of 5.6 or 16.7 mmol/L glucose, as appropriate, with a range of test peptide concentrations (10^{-12} to 10^{-6} M). The effects of PYY(1-36) and PYY(3-36) on alanine and GLP-1 induced insulin secretion was also examined in BRIN BD11 cells. After 20 min incubation, buffer was removed from each well and aliquots stored at -20°C prior to determination of insulin by radioimmunoassay (Flatt and Bailey, 1982). To determine potential NPY receptor involvement, BRIN BD11 cells were incubated at 16.7 mM glucose with PYY(1-36) or PYY(3-36) (10^{-8} and 10^{-6} M) and either the specific NPY1 receptor antagonist BVD-10 (10^{-6} M; Tocris Bioscience) or the specific NPY2 receptor antagonist BIIE 0246 (10^{-6} M; Tocris

Bioscience), and insulin secretion determined as described above. In addition, to determine potential mechanisms of action, membrane potential and intracellular Ca^{2+} were also determined following treatment with test peptides (10^{-6} M) in BRIN BD11 cells using a Flexstation scanning fluorometer (FLIPR Calcium 5 assay kit, FLIPR membrane potential assay kit, Molecular devices, Sunnyvale, USA) as described previously (Miguel et al., 2004). Furthermore, effects of PYY(1-36) and PYY(3-36) on GLP-1 mediated elevations of intracellular cAMP were assessed in BRIN BD11 cells using a Parameter cAMP assay (R&D Systems, Abingdon, UK), according to the manufacturer's instructions. In a separate series, pancreatic islets were isolated from lean control C57BL/6 mice by collagenase digestion, as described previously (Pathak et al., 2015). Insulin secretion was determined as above, but with a 60 min test incubation period. In one series of experiments the actions of GLP-1, PYY(1-36) and PYY(3-36) were examined in the absence and presence of the DPP-4 inhibitor sitagliptin (500 nM, ApexBio Technology, Houston, TX, USA). Following removal of the test solution, 200 μ l of acid-ethanol solution (1.5% (v/v) HCl, 75% (v/v) ethanol, 23.5% (v/v) H_2O) was added for overnight extraction of cellular insulin. All samples were stored at -20°C for measurement of insulin concentrations by radioimmunoassay (Flatt and Bailey, 1982).

2.5 Islet histology studies in insulin-deficient and insulin-resistant diabetic mice

To induce insulin-deficient diabetes, multiple low dose streptozotocin (50 mg/kg body weight, i.p.) in 0.1 M sodium citrate buffer (pH 4.5) or saline vehicle (0.9% w/v NaCl, i.p.) was injected daily (13:00 h, n=6, fasted for 4 h) over a period of 5 days in C57BL/6 mice (Vasu et al., 2014). Pancreatic tissues were excised five days after the final injection. To induce insulin-resistant diabetes, hydrocortisone was injected i.p. (70 mg/kg body weight in saline, n=6) once daily over a period of 10 days in C57BL/6 mice, and pancreatic tissues extracted at the end of the treatment period (Vasu et al., 2014). All pancreatic tissues were immediately fixed in 4% PFA

for 48 h at 4°C and subsequently dehydrated using a series of increasing strength ethanol solutions and processed for embedding in paraffin wax using an automated tissue processor (Leica TP1020, Leica Microsystems, Nussloch, Germany), as described previously (Vasu et al., 2013; Moffett et al., 2013). Tissue blocks were sectioned (8 µm) using a Shandon Finesse 325 microtome (Thermo Scientific, Hemel Hempstead, UK) and picked for staining at intervals of 10 sections. After deparaffinising, sections were rehydrated using a series of decreasing strength ethanol solutions. Antigen retrieval was carried out using a citrate buffer (pH 6.0) at 94°C for 20 min, sections were then blocked using 2% BSA and incubated overnight at 4°C with appropriate primary antibody (Table 1). The slides were then incubated with appropriate secondary antibodies (Alexa Fluor® 594 for red and Alexa Fluor® 488 for green; Table 1) and stained with nuclear DAPI staining. Importantly, we confirmed the specificity of our PYY antibody in blocking experiments using the native PYY(1-36) peptide, and no cross-reactivity with PP was observed. Slides were mounted with anti-fade mounting medium and viewed using a fluorescent microscope (Olympus System Microscope, model BX51; Southend-on-Sea, UK). The slides were photographed using a DP70 camera adapter system (Vasu et al., 2013; Moffett et al., 2013). All staining procedures and image analysis were carried out in a blinded manner. Approximately 150 islets were analysed per group. Islet parameters were determined using the ‘closed polygon’ tool in Olympus Cell[^]F analysis software. PYY expression in alpha/PP/somatostatin cells was determined by counting cells with PYY and glucagon/PP/somatostatin expression and expressed as % of total alpha/PP/somatostatin cells. Areas of insulin and glucagon positive cells were calculated using the closed Polygon tool in Cell[^]F software, and expressed as total islet area, µm².

2.6 Beta-cell proliferation and cellular stress studies

To assess the effects of PYY(1-36) and PYY(3-36) on rodent BRIN-BD11 and human 1.1B4 cell proliferation, cells were seeded at a density of 150,000 cells per well and cultured overnight in the presence of PYY peptides (10^{-6} M), with GLP-1 (10^{-6} M) as a positive control. Cells were rinsed with PBS and fixed using 4 % paraformaldehyde. After antigen retrieval with citrate buffer at 95°C for 20 min, tissue was blocked using 2% BSA for 45 minutes. The slides were then incubated with rabbit anti-Ki-67 primary antibody, and subsequently with Alexa Fluor® 594 secondary antibody. Slides were viewed using fluorescent microscope (Olympus System Microscope, model BX51; Southend-on-Sea, UK) and photographed by DP70 camera adapter system. Proliferation frequency was determined in a blinded fashion and expressed as % of total cells analysed. Approximately 150 cells per replicate were analysed. For analysis of ability of PYY(1-36) and PYY(3-36) to protect against streptozotocin-induced DNA damage, BRIN-BD11 and 1.1B4 cells were seeded as above. Cells were then exposed to streptozotocin (5 mM) in the presence or absence of test peptides (10^{-6} M) for 2 hours, with GLP-1 (10^{-6} M) as positive control. Cells were then harvested and a comet assay was performed as described previously (Lees-Murdock et al., 2004). Resulting gels were stained using DAPI (4', 6 – diamidino – 2 – phenylindole) (100 µg/ml) and slides were viewed under appropriate filter using an Olympus fluorescent microscope. Comet score software (Version 1.5) was used for the analysis of % tail DNA (100 cells per gel) and olive tail moment.

2.7 Biochemical analyses

Blood samples were collected from the cut tip on the tail vein of conscious mice at the time points indicated in the Figs. Blood glucose was measured directly using a hand-held Ascencia Contour blood glucose meter (Bayer Healthcare, Newbury, Berkshire, UK). For plasma insulin analysis, blood samples were collected into chilled fluoride/heparin glucose micro-centrifuge tubes (Sarstedt, Numbrecht, Germany) and immediately centrifuged using a Beckman

microcentrifuge (Beckman Instruments, Galway, Ireland) for 1 min at 13,000 x *g* and stored at -20 °C, prior to determination of insulin concentrations by a modified insulin radioimmunoassay (Flatt and Bailey, 1982).

2.8 Statistical analysis

Statistical analyses were performed using GraphPad PRISM software (Version 5.0). Values are expressed as mean±S.E.M. Comparative analyses between groups were carried out using a One-way ANOVA with Berferroni post hoc test or student's unpaired t-test, as appropriate. The difference between groups was considered significant if $p < 0.05$.

3. Results

3.1 Expression of PYY and NPY receptor mRNA in BRIN BD11 beta-cells, isolated mouse islets and immortalised human beta-cell pseudoislets

As shown in Figure 1, PYY was expressed in mouse islets at slightly increased levels compared to PP mRNA expression (Fig. 1A). Glucagon mRNA expression was significantly ($p < 0.001$) higher than the β -actin housekeeping gene, whereas PP and PYY expression was significantly ($p < 0.05$) lower (Fig. 1A). In terms of NPY receptor expression, all data were normalised to β -actin expression and then comparisons made against the classic islet receptor, *GIPR* (Fig. 1B-D). As such, *NPY1R* mRNA expression was particularly abundant in all experimental models examined, with comparable or increased when compared to expression of *GIPR* (Fig. 1B-D). *NPYR5* was undetectable in cultured human 1.1B4 cells (Fig. 1D), and was also expressed at a relatively low ($p < 0.05$) levels in isolated mouse islets (Fig. 1B), but at higher ($p < 0.01$) levels in BRIN BD11 cells. In addition, *NPY2R* expression was also diminished (p

< 0.01 to $p < 0.001$) in mouse islets and 1.1B4 beta-cells compared to GIP R (Fig. 1B,D), with relatively similar levels to *GIPR* in BRIN BD11 cells (Fig. 1C). In general, the expression profile of NPYR1, 2 and 4 was similar in mouse islets and immortalised human 1.1B4 beta-cells (Fig. 1B,D). Notably, expression levels of all NPYR's and *GIPR* were elevated in BRIN BD11 cells when compared to the other experimental cellular models (Fig. 1C). In addition, both mouse islets and 1.1B4 cells had comparatively high expression levels on *NPYR4* compared to other NPYR's, whereas this receptor subtype was expressed at relatively low levels in BRIN BD11 cells (Fig. 1C).

3.2 Effects of PYY(1-36) and PYY(3-36) on glucose tolerance and food intake in mice

Administration of 25 nmol/kg PYY(1-36) or PYY(3-36) to normal mice in combination with glucose had no significant effect on blood glucose levels (Fig. 2A,B). In agreement, corresponding plasma insulin levels were not significantly altered by PYY(1-36) or PYY(3-36) (Fig. 2C,D). However, whilst PYY(1-36) did not affect feeding behaviour in overnight fasted mice, PYY(3-36) significantly ($p < 0.05$) reduced food intake at 120, 150 and 180 min post-injection (Fig. 2E).

3.3 Effects of PYY(1-36) and PYY(3-36) on insulin release from rodent BRIN BD11 beta-cells, isolated mouse islets and immortalised human 1.1B4 beta-cells

At both 5.6 and 16.7 mM glucose, KCl (30 mM), alanine (10 mM) and GLP-1 (10^{-6} M and 10^{-8} M) significantly ($p < 0.001$) increased insulin release from BRIN BD11 cells, when compared to respective glucose controls (Fig. 3A,B). In contrast, all concentrations of PYY(1-36) or PYY(3-36) examined (10^{-10} to 10^{-6} M) did not significantly affect insulin release at 5.6 mM

glucose (Fig. 3A). However, both forms of PYY significantly inhibited glucose-stimulated (16.7 mM) insulin release, with the exception of 10^{-10} M PYY(3-36) (Fig. 3B). Essentially similar observations were made in 1.1B4 cells, including inhibition of glucose-stimulated insulin release (Fig. 3C). Incubation of PYY(1-36) with the NPY1 specific receptor antagonist BVD-10 completely reversed ($p < 0.001$) the insulinostatic effect of PYY(1-36) at 16.7 mM glucose, whereas NPY2 receptor antagonism had no effect (Fig. 3D). Interestingly, BVD-10, and the NPY2 specific receptor antagonist, BII0246, both inhibited ($p < 0.05$ to $p < 0.01$) the insulinostatic actions of PYY(3-36) in BRIN BD11 cells at 16.7 mM glucose (Fig. 3D). PYY(1-36) and PYY (3-36) also significantly ($p < 0.05$) inhibited GLP-1 induced insulin release from BRIN BD11 cells (Fig. 3E). Both forms of PYY significantly ($p < 0.05$ to $p < 0.001$) inhibited alanine-induced insulin release at concentrations of 10^{-9} to 10^{-6} M (Fig. 3F), but had no effect on alanine-induced insulin secretion at lower concentrations of 10^{-10} M (Fig. 3F). In harmony with findings from immortalised rodent and human beta-cells, both PYY(1-36) and PYY(3-36) (10^{-8} to 10^{-6} M) inhibited ($p < 0.01$ to $p < 0.001$) glucose-induced insulin secretion from isolated mouse islets (Fig. 3G). Primary islets were also used to assess the effects of DPP-4 inhibition with 500 nM sitagliptin on the insulin-releasing actions of exogenous GLP-1 and PYY peptides (Fig. 3H). Sitagliptin enhanced ($p < 0.05$) insulin release at 5.6 mM (possibly by inhibiting degradation of islet GLP-1 and GIP (Vasu et al., 2014; Moffett et al., 2015) and potentiated ($p < 0.05$) GLP-1-induced insulin secretion (Fig. 3H). Most notably, sitagliptin increased ($p < 0.05$) the inhibitory effect of PYY(1-36) on alanine-induced insulin release without affecting the inhibitory actions of PYY(3-36) (Fig. 3H).

3.4 Effects of PYY(1-36) and PYY(3-36) on membrane potential, intracellular Ca^{2+} and cAMP levels in rodent BRIN BD11 cells

Alanine (10 mM) significantly ($p < 0.05$ to $p < 0.001$) increased cell membrane potential and $[Ca^{2+}]_i$ in BRIN BD11 cells (Fig. 4A-D). The two forms of PYY partially inhibited ($p < 0.05$ to $p < 0.001$) the positive effects of alanine on both membrane potential and $[Ca^{2+}]_i$ (Fig. 4B,D). As expected, GLP-1 and forskolin markedly ($p < 0.001$) increased intracellular cAMP generation, whilst PYY(1-36) and PYY(3-36) significantly ($p < 0.001$) inhibited GLP-1 induced elevations of cAMP (Fig. 4E). The batch of forskolin tested in these experiments evoked a relatively small increase of cAMP.

3.5 Effects of insulin-deficiency and insulin-resistance on islet architecture and morphology

Fig. 5A shows representative images of pancreatic islets from saline, streptozotocin and hydrocortisone treated C57BL/6 mice. Streptozotocin significantly reduced beta-cell area ($p < 0.01$) but did not alter overall islet area (Fig. 5B,C). This appeared to be partly due to a marked ($p < 0.001$) increase of both alpha and PP cell areas compared to control mice (Fig. 5D,E). In contrast, hydrocortisone significantly ($p < 0.001$) enhanced both islet and beta-cell areas, but had no obvious effect on alpha and PP cell areas (Fig. 5B-E). Neither intervention affected PYY or delta cell area (Fig. 5F,G). Further to this, streptozotocin reduced ($p < 0.05$), and hydrocortisone increased ($p < 0.01$), the number of islets compared to saline control mice (Fig. 5H). Interestingly, islet size distribution was unchanged in streptozotocin treated mice, whereas hydrocortisone significantly ($p < 0.001$ and $p < 0.01$; respectively) increased the proportion of medium and large sized islets, and reduced ($p < 0.001$) the number of smaller sized islets (Fig. 5I). Representative images showing co-localisation of PYY with glucagon, PP or somatostatin in islets of control, streptozotocin and hydrocortisone treated mice are shown in Fig. 6A. Despite lack of effect of treatments on overall PYY cell area (Fig. 5G), distinct differences in islet cell populations expressing PYY were revealed. As such, hydrocortisone increased ($p <$

0.05) co-localisation of PYY with glucagon (Fig. 6B), while streptozotocin decreased ($p < 0.001$ and $p < 0.05$; respectively) co-localisation of PYY with somatostatin and PP positively stained cells (Fig. 6C,D).

3.6 Effects of PYY(1-36) and PYY(3-36) on immortalised rodent BRIN BD11 and human 1.1B4 cell proliferation

Exposure of BRIN BD11 or 1.1B4 cells to GLP-1, PYY(1-36) or PYY(3-36) at 10^{-6} for 16 h significantly ($p < 0.05$ to $p < 0.01$) increased proliferation frequency compared to control cultures (Fig. 7A,B). Intriguingly, the proliferative effects of GLP-1 and PYY(1-36) in BRIN BD11 cells appeared to be linked to decreased ($p < 0.01$) gene expression of *Crebp*, and unaltered *Foxo1* and *Pdx1* expression (Table 2). Although reductions in *Pdx1* gene expression levels were noted with both GLP-1 and PYY(1-36) treatment, this failed to reach significance (Table 2). PYY(3-36) had no significant effect on the expression levels of *Crebp*, *Foxo1* or *Pdx1* (Table 2). Representative images of Ki67 stained BRIN BD11 and 1.1B4 cells under each culture condition are shown in Fig. 7C.

3.7 Protective effects of PYY(1-36) and PYY(3-36) on streptozotocin-induced DNA damage in immortalised rodent BRIN BD11 and human 1.1B4 cells

Streptozotocin (5 mM) significantly ($p < 0.001$) decreased both BRIN BD11 and 1.1B4 cell viability, which was almost fully countered by co-culture with GLP-1 (10^{-6} M), and partially countered in 1.1B4 cells by PYY(1-36) and PYY(3-36) (Fig. 8A,B). Streptozotocin also significantly ($p < 0.001$) increased % tail DNA and olive tail moment in both beta-cell lines (Fig. 8C-F). Culture of BRIN BD11 and 1.1B4 cells with either GLP-1, PYY(1-36) or PYY(3-

36) reversed the detrimental effects of streptozotocin on % tail DNA and olive tail moment (Fig. 8C-F). However, notably in human 1.1B4 cells the beneficial effects of PYY(1-36) and PYY(3-36) on % tail DNA and olive tail moment were visibly superior to GLP-1 (Fig. 8D,F). Indeed, both forms of PYY returned % tail DNA to control levels following streptozotocin insult, whereas cells treated with GLP-1 still had significantly ($p < 0.001$) increase % tail DNA when compared to control cultures (Fig. 8D). In terms of gene expression, streptozotocin significantly ($p < 0.05$) reduced mRNA expression of *Bcl2* without effecting transcription of *Nfkb1* and *Bax* in BRIN BD11 cells (Table 2). GLP-1 and PYY(3-36) treatment increased *Bcl2* mRNA levels, and expression levels were not significantly different from control cultures (Table 2). PYY(1-36) treatment did not alter the reduced *Bcl2* expression levels induced by streptozotocin (Table 2). In addition, PYY(1-36) reduced ($p < 0.05$) *Nfkb1* mRNA levels, an effect which was not observed with any of the other treatment modalities (Table 2). None of the culture conditions significantly affected *Bax* expression levels (Table 2). Representative comet images from each culture condition in BRIN BD11 and 1.1B4 cells are shown in Fig. 8G.

4. Discussion

The most widely accepted biological function of PYY, secreted from intestinal L-cells in response to feeding, is as an anorectic hormone that reduces food intake and subsequently body weight (Batterham et al., 2002). However, the expression and synthesis of PYY in murine islet cells, as shown here, and previously (Ali-Rachedi et al., 1984; Bottcher et al., 1993), suggests a possible important autocrine or paracrine role for this peptide hormone in regulating islet function.

As expected (Batterham and Bloom, 2003), PYY(3-36) significantly reduced food intake in overnight fasted mice in the current study, whereas PYY(1-36) was devoid of appetite-suppressive actions. This effect is linked to likely selectivity and high affinity of PYY(3-36) for NPY2 receptors, whereas PYY(1-36) is believed to bind to all NPY receptors (NPY1, 2, 4 and 5) with similar affinity (Grandt et al., 1992). Neither PYY(1-36) nor PYY(3-36) altered circulating glucose or insulin levels in mice, suggesting that PYY released from the gut has little direct input on blood glucose control. However, both peptides evoked a notable reduction in glucose-stimulated insulin secretion from immortalised BRIN BD11 and human beta-cells, as well as isolated mouse islets. This apparent discrepancy between *in vitro* and *in vivo* observations may require further study, but in our view could simply be related to differences in concentrations of peptides exposed directly to islet cells. An insulinostatic action is largely in agreement with others (Szecowwka et al., 1983; Bottcher et al., 1989; Sam et al., 2012), and importantly observed at concentrations of PYY greater than those encountered in the circulation (Murphy and Bloom, 2006). Nevertheless, such concentrations could potentially be encountered locally within islets as suggested by widespread PYY protein expression in alpha, delta and PP cells together with demonstration of mRNA for PYY at levels greater than the classical islet peptide PP. In contrast, it has recently been shown that chronic exposure of diabetic rat isolated islets to PYY restores insulin secretory function (Ramracheya et al., 2016). Moreover, in one report peripheral PYY(3-36) administration was revealed to augment insulin release and improve glucose homeostasis, albeit through an action believed to be dependent on GLP-1 secretion (Chandarana et al., 2013).

Clearly further studies are required to fully assess the longer-term impact of both PYY(1-36) and PYY(3-36) on beta-cell insulin secretory function, but our data confirm an inhibitory insulintropic effect upon acute exposure of beta-cells to either form of PYY. In agreement, we detected co-localisation of PYY with all murine islet cell types, except beta-

cells. This is consistent with previous observations that PYY is not naturally expressed in adult mouse beta-cells (Upchurch et al., 1994; Sam et al., 2012). However, indicative of a role in beta-cell function, mouse islets and both immortalised human and rodent pancreatic beta-cells expressed NPY receptors at levels quite similar to GIPR, in accordance with GPCR screening studies (Amisten et al., 2013). Notably, the expression of all NPY receptors was elevated in BRIN BD11 cells, when compared to both mouse islets and immortalised human 1.1B4 beta-cells, which may require further investigation and could also impact on some of our current observations. Others have struggled to evidence expression of NPY2 receptors in pancreatic islets cells (Chandarana et al., 2013), but we have clearly shown detectable amounts in isolated mouse islets, as well as immortalised rodent and human beta-cells in the present study.

Quantification of mouse islet and human 1.1B4 cell NPY receptor mRNAs indicated that NPY1R and NPY4R were most abundant, with much lower expression of NPY2R and NPY5R. In fact, the latter was undetectable in immortalised human beta-cells. Of the various receptor subtypes, the NPY1 receptor has been suggested by others to be particularly important in regulation of islet function (Morgan et al., 1998; Amisten et al., 2013). Whilst BRIN BD11 cells expressed high levels of NPY1R, the expression of NPY2R and NPY5R was also significant. These differences in NPY receptor mRNA levels between the cellular models used is interesting and requires further investigation. Nonetheless, the effects of PYY(1-36) and PYY(3-36) on insulin secretion were remarkably similar across all *in vitro* systems, arguing against a key role for NPY5 and possibly 4 receptors. Since PYY(3-36) is considered to be selective for NPY2 receptors, these results together with present demonstration of the NPY2 receptor in various beta-cell preparations suggests that islet effects of PYY(3-36) may be mediated through this receptor subtype. However, we cannot entirely rule out mediation of effects via NPY1 receptors which bind PYY(3-36), albeit with weaker affinity (Karra et al., 2009). Indeed, our preliminary *in vitro* studies in BRIN BD11 cells with selective NPY1 and 2

receptor antagonists support this concept. As such, beta-cell actions of PYY(1-36) appear to be solely mediated by NPY1 receptors, whereas PYY(3-36) actions were dependent on interaction with both NPY1 and 2 receptors. Regrettably, data from NPY1 and 2 receptor knockout mice is clouded through significant influence of genetic background on phenotype (Zambello et al., 2011), and the possibility that NPY1 receptors may regulate the expression of NPY2 receptors (Wittmann et al., 2005).

Investigation of possible insulinostatic mechanisms of action of PYY revealed inhibition of the insulin-releasing effects of GLP-1 and alanine, together with reductions of GLP-1 induced cAMP production (Nieuwenhuizen et al., 1994), and alanine-induced changes of BRIN BD11 beta-cell membrane potential and $[Ca^{2+}]_i$. Thus, PYY appears to uncouple various insulin secretory signal transduction pathways in beta-cells. Since PYY(1-36) is a substrate for DPP-4 which is present in islets (Omar et al., 2014) it is not clear whether conversion to PYY(3-36) is significant in the normal intra-islet effects of PYY. However, our preliminary studies utilising a DPP-4 inhibitor suggest that PYY(1-36) is degraded *in vitro* by mouse islets and that inhibition of truncation to PYY(3-36) enhanced its action. Although, in our hands PYY(3-36) appeared to possess similar insulinostatic properties as PYY(1-36), these data suggest that PYY(1-36) and activation of receptors other than NPYR2 may be particularly important. Irrespective of the receptor subtype and mechanism involved, the insulinostatic actions of both forms of PYY could represent a physiologically relevant brake for locally produced islet PYY to limit excessive beta-cell stimulation, potential beta-cell exhaustion and ultimately loss of beta-cell mass. This is potentially therapeutically relevant because beta-cell rest has been shown to preserve long-term beta-cell function and improve overall enduring glycaemic control (Brown and Rother, 2008; Pathak et al., 2015), and is plausible mechanism for PYY-induced beneficial pancreatic actions. Therefore, we next assessed PYY islet

expression in both streptozotocin and hydrocortisone treated mice, to evaluate potential impact of locally produced PYY in situations of both islet cell destruction and growth, respectively.

Streptozotocin and hydrocortisone treatment induced characteristic opposing changes in murine islet morphology, consistent with previous observations (Vasu et al., 2014). As such, streptozotocin reduced islet number and beta-cell area (Vasu et al., 2014), but also induced marked increases in the alpha and PP cell areas with no effect on delta cells, confirming that these cell types are resistant to streptozotocin-induced toxicity (Rombout et al., 1987; Vasu et al., 2015). Co-localisation studies in streptozotocin mice revealed that PP and delta cells had decreased co-expression with PYY, but alpha cell co-localisation was unaltered. Considering that alpha cell area was dramatically increased in these mice, with no change in PYY cell area, the data suggests a relative increase in PYY co-localisation with alpha cells. Interestingly, it has previously been indicated that alpha-cells may act as progenitors for beta-cells, particularly under situations of severe beta-cell loss (Chung and Levine, 2010). In agreement, hydrocortisone increased PYY co-localisation with glucagon, and was associated with an expansion of beta-cell mass. Use of selective PYY antagonists could help determine the exact role of PYY signalling on endocrine islet cell morphology following intervention with streptozotocin or hydrocortisone. Moreover, in the present study both PYY(1-36) and PYY(3-36) significantly augmented beta-cell proliferation by immortalised rodent and human beta-cells, at a comparable level to GLP-1 (Baggio and Drucker, 2007; Campbell and Drucker, 2013). Mitogenic actions of PYY have previously been documented (Kazanjian et al., 2003), and linked to activation of phospholipase C and mitogen-activated protein kinases, leading to phosphorylation of ERK1/2 which is directly involved in gene transcription and cell proliferation (Hansel et al., 2001; Howell et al., 2007; Persaud and Bewick, 2014). Interestingly, neither form of PYY affected transcription of *Foxo1* and *Pdx1*, transcription factors known to be involved in the maintenance of beta-cell function and survival (Bernardo

et al., 2008). It should also be recognised that proliferation studies were conducted in immortalised beta-cell lines that inherently would be in a hyperproliferative state.

It has been proposed that PYY mainly protects against beta-cell loss by preventing apoptosis, rather than directly increasing proliferation (Sam et al., 2012). In the current study, both PYY(1-36) and PYY(3-36) partially protected BRIN BD11 and 1.14B beta-cells from streptozotocin-induced DNA damage, to a similar or superior degree, as observed with GLP-1 (Thiriet et al., 2005). This is consistent with the notion that NPY receptor activation increases PKC epsilon activity, which has direct inhibitory role in apoptosis (Mannon, 2002). As such, a long-acting, NPY1 and NPY2 receptor specific, PYY analogue has been shown to reverse streptozotocin-induced beta-cell loss in mice (Sam et al., 2012). Surprisingly, streptozotocin treatment resulted only in a decrease of *Bcl2* transcription in our study, with no effect on *Bax* or *Nfkb1* expression, which could be linked to the duration of the study or the streptozotocin treatment regimen employed. GLP-1 and PYY(3-36) reversed this detrimental effect on *Bcl2* transcription, whereas co-culture with PYY(1-36) actually reduced *Nfkb1* expression. Thus, the ability of PYY(1-36) to protect pancreatic beta-cells from apoptosis (Sam et al., 2012) could be linked to modulation of *Nfkb1* related cell signalling pathways, which is intriguing and requires further detailed study (Eizirik et al., 2001). In addition, it would also be useful to determine if PYY(1-36) alters translocation of *Nfkb1* to the nucleus, a key feature of *Nfkb1* signalling (Baeuerle, and Baltimore, 1996).

Taken together, our combined observations along with recently published literature (Sam et al., 2012; Persaud and Bewick, 2014; Shi et al., 2015), provides evidence that PYY plays an essential role in regulating beta-cell function and preserving beta-cell mass. Given that beta-cell dysfunction and decreased beta-cell mass are classic features of both type 1 and type 2 diabetes (Thorens, 2013), our data suggest that NPY receptor modulation could represent a novel target for the treatment, or prevention, of diabetes.

Author Contributions

DK, SV and RCM contributed to conduct/data collection, analysis and writing of the manuscript. NI and PRF contributed to study design, analysis and writing of the manuscript. All authors approved the final version of the manuscript. No potential conflict of interests relevant to this article were reported.

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Table 1. Target, host and source of primary and secondary antibodies employed for immunofluorescent islet histology studies

Primary antibodies

Target	Host	Dilution	Source
Insulin	Mouse	1:500	Abcam, ab6995
Glucagon	Guinea pig	1:200	Raised in-house PCA2/4
PYY	Rabbit	1:500	Abcam, ab22663
PP	Goat	1:200	Abcam, ab77192
SST	Rat	1:500	Biorad, 8330-009
Ki67	Rabbit	1:200	Abcam, ab15580

Secondary antibodies

Target	Host	Reactivity	Dilution	Fluorescent dye and Source
IgG	Goat	Mouse	1:400	Alexa Flour 594, Invitrogen, UK
IgG	Goat	Guinea pig	1:400	Alexa Flour 488, Invitrogen, UK
IgG	Goat	Rabbit	1:400	Alexa Flour 594, Invitrogen, UK
IgG	Goat	Rat	1:400	Alexa Flour 488, Abcam
IgG	Donkey	Goat	1:400	Alexa Flour 488, Invitrogen, UK

Table 2 Effects of PYY(1-36) and PYY(3-36) on expression of genes involved in proliferation and apoptosis.

Proliferation	Control	Streptozotocin (5 mM)	GLP-1 (10⁻⁶ M)	PYY(1-36) (10⁻⁶ M)	PYY(3-36) (10⁻⁶ M)
1. <i>Foxo1</i>	100±14.09	ND	62.5±19.2	89.5±16.2	126.3±11.1
2. <i>Pdx1</i>	100±15.16	ND	87.3±60.0	50.0±16.1	74.7±15.8
3. <i>Crebp</i>	100±10.75	ND	20.5±19.0**	20.3±16.4**	70.5±12.9
Apoptosis	Control	Streptozotocin (5 mM)	GLP-1 (10⁻⁶ M)	PYY(1-36) (10⁻⁶ M)	PYY(3-36) (10⁻⁶ M)
1. <i>Nfkb1</i>	100±14	94.2±16.1	111.3±12.2	43.9±15*	50±16.3
2. <i>Bax</i>	100±9.8	67±12.4	67.1±15.4	76.3±19.7	102.3±13.9
3. <i>Bcl2</i>	100±20.5	43.4±9.9*	57.5±15.9	24±12.9*	49.1±20.6

Gene expression for proliferation was assessed in BRIN BD11 beta-cells cultured with PYY peptides or GLP-1 (10⁻⁶ M) for 16 h. Gene expression for apoptosis was similarly assessed in BRIN BD11 cells exposed to 5 mM streptozotocin. Values are mean ± SEM (n=4). Gene expression was normalized to *Actb* expression. **p* < 0.05, ***p* < 0.01 compared to untreated control. ND, not determined.

Figure Legends

Fig. 1. mRNA expression of *PYY* and *NPYR*. (A) *PYY* mRNA expression was compared to classical islet hormones glucagon, somatostatin and PP in mouse islets. (B-D) *NPY* receptor mRNA expression in (B) mouse islets, as well as immortalised (C) rodent and (D) human beta-cell lines. Values are mean \pm SEM (n = 4-6). (A) * p < 0.05, *** p < 0.001 compared to β -actin mRNA expression. (B) * p < 0.05, ** p < 0.01, *** p < 0.001 compared to *GIPR* mRNA expression. All mRNA expression was normalized to *Actb*/ACTB expression.

Fig. 2. Acute effects of *PYY*(1-36) and *PYY*(3-36) on glucose tolerance, insulin response to glucose and food intake in overnight fasted mice. (A) Blood glucose and (C) plasma insulin levels were assessed immediately before and after intraperitoneal administration of *PYY*(1-36) or *PYY*(3-36) (25 nmol/kg bw) together with glucose (18 mmol/kg bw). Respective (B) blood glucose and (C) plasma insulin area under curve data is also shown. (E) Cumulative food intake was assessed after intraperitoneal administration of saline vehicle (0.9% NaCl), *PYY*(1-36) or *PYY*(3-36) (25 nmol/kg bw) in overnight fasted mice. Values are mean \pm SEM (n=6 mice). * p < 0.05 compared to saline treated mice.

Fig. 3. Effects of *PYY*(1-36) and *PYY*(3-36) on insulin release from immortalised rodent BRIN BD11 beta-cells, human 1.1B4 beta-cells and isolated mouse islets. (A,B) BRIN BD11 or (C) 1.1B4 cells were incubated with either 5.6 or 16.7 mM glucose, as appropriate, and the effects of *PYY* peptides (10^{-10} – 10^{-6} M) determined. (D) Effects of selective *NPY*1 (BVD-10) and *NPY*2 (BII0246) receptor antagonists on *PYY*(1-36)- and *PYY*(3-36)-mediated insulin release from rodent BRIN BD11 beta-cells. Effects of *PYY* peptides on (E) GLP-1 (10^{-6} M) or (F)

alanine (10 mM) induced insulin release were also determined using BRIN BD11 cells. (G) Effects of PYY peptides (10^{-8} – 10^{-6} M) in isolated mouse islets. (H) Effects of sitagliptin (500 nM) on the insulin secretory actions of GLP-1 (10^{-6} M) and ability of PYY peptides (10^{-6} M) to inhibit alanine-induced insulin secretion in isolated mouse islets. (A-F) Values are mean \pm SEM (n=8). * p < 0.05, ** p < 0.01, *** p < 0.001 compared to respective control. Δp < 0.05, $\Delta\Delta p$ < 0.01 compared to GLP-1. (G,H) Values are mean \pm SEM (n=4). * p < 0.05, ** p < 0.01, *** p < 0.001 compared to 16.7 mM glucose. Δp < 0.05 to peptide in absence of sitagliptin. $\phi\phi\phi p$ < 0.001 compared to alanine.

Fig. 4. Effects of PYY(1-36) and PYY(3-36) on membrane potential, $[Ca^{2+}]_i$ and GLP-1-induced cAMP production in rodent BRIN BD11 cells. (A,C) Cells were incubated with 5.6 mM glucose in the presence of alanine (10 mM) or PYY peptides (10^{-6} M) and membrane potential or $[Ca^{2+}]_i$ assessed over a 5 minute period, with alanine (10 mM) as positive control. (B,D) area under curve data is also shown. (E) BRIN BD11 cells were incubated (20 min) with GLP-1 (positive control; 10^{-6} M) in the absence and presence of PYY peptides (10^{-6} M) and intracellular cAMP was assessed by ELISA. Values are mean \pm SEM (n=6). ** p < 0.01, *** p < 0.001 compared to 5.6 mM glucose. Δp < 0.05, $\Delta\Delta p$ < 0.01, $\Delta\Delta\Delta p$ < 0.001 compared to respective positive control.

Fig. 5. Effects of streptozotocin and hydrocortisone treatment on *in vivo* islet morphology and cells expressing PYY. C57BL/6 mice received daily injections of streptozotocin (50 mg/kg bw) or hydrocortisone (70 mg/kg bw) for 5 or 10 days respectively before examination of pancreatic histology on day 10. (A) Representative islet images showing insulin (red) and glucagon (green) staining in islets of control, streptozotocin and hydrocortisone treated mice. Nuclei are

demonstrated using DAPI staining (blue). Arrows indicate glucagon positive cells. (B) Islet, (C) beta-cell, (D) alpha cell, (E) PP cell, (F) delta cell and (G) PYY cell areas, as well as (H) number and (I) size distribution of islets, were determined using the ‘closed polygon’ tool in Olympus Cell[^]F analysis software. Values are mean \pm SEM (n=6 mice). * p < 0.05, ** p < 0.01, *** p < 0.001 compared to control mice.

Fig. 6. Effects of streptozotocin and hydrocortisone treatment on co-localisation of PYY with glucagon, PP and somatostatin. C57BL/6 mice received daily injections of streptozotocin (50 mg/kg bw) or hydrocortisone (70 mg/kg bw) for 5 or 10 days respectively before examination of pancreatic histology on day 10. (A) Representative images showing PYY (red) staining with glucagon, PP and somatostatin (green) in islets of control, streptozotocin and hydrocortisone treated mice. Nuclei are demonstrated using DAPI staining (blue). Arrows indicate cells that are positive for both PYY and glucagon, PP or somatostatin. Quantification of co-localization of PYY with (B) glucagon, (C) PP and (D) and somatostatin is also shown. Values are mean \pm SEM (n=6 mice). * p < 0.05, *** p < 0.001 compared to control mice.

Fig. 7. Effects of PYY(1-36) and PYY(3-36) on immortalised rodent BRIN BD11 and human 1.1B4 cell proliferation. (A,B) Proliferation frequency in (A) BRIN BD11 and (B) 1.1B4 cells cultured with PYY or GLP-1 (10^{-6} M) for 16 h. (C) Representative images showing proliferating beta-cells in the presence (16 h) of PYY or GLP-1 (10^{-6} M). Arrows indicate proliferating cells. Values are mean \pm SEM (n=4). * p < 0.05, ** p < 0.01 compared to control.

Fig. 8. Effects of PYY(1-36) and PYY(3-36) on protection of rodent BRIN BD11 cells from streptozotocin induced cellular stress. (A,B) Cell viability, (C,D) % tail DNA and (E,F) olive tail moment were assessed in response to 16 h exposure to 5 mM streptozotocin with and without co-culture with PYY or GLP-1 (10^{-6} M) in (A,C,E) BRIN BD11 and (B,D,F) 1.1B4 beta-cells. (G) Representative images showing comets of control, streptozotocin alone and in combination with PYY or GLP-1 peptides (10^{-6} M) in both cell types. Arrows indicate cells with comet tails. Values are mean \pm SEM (n=4). *** p < 0.01 compared to control. $\Delta\Delta p$ < 0.01, $\Delta\Delta\Delta p$ < 0.001 compared to streptozotocin treated cells.

Figure 1

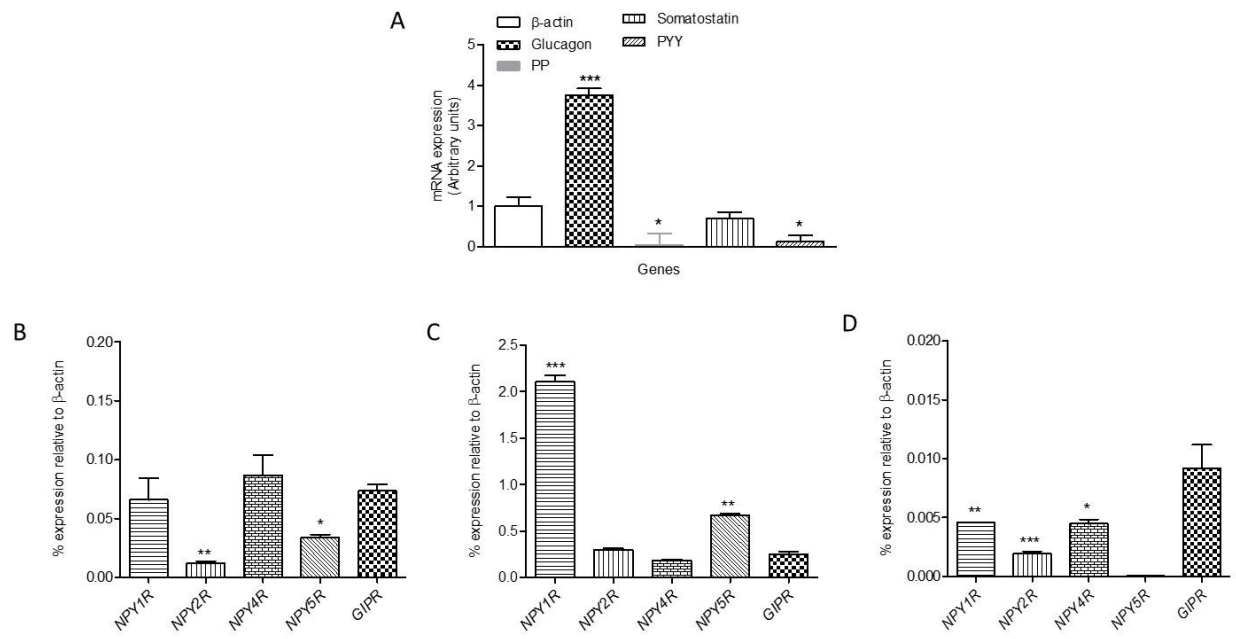


Figure 2

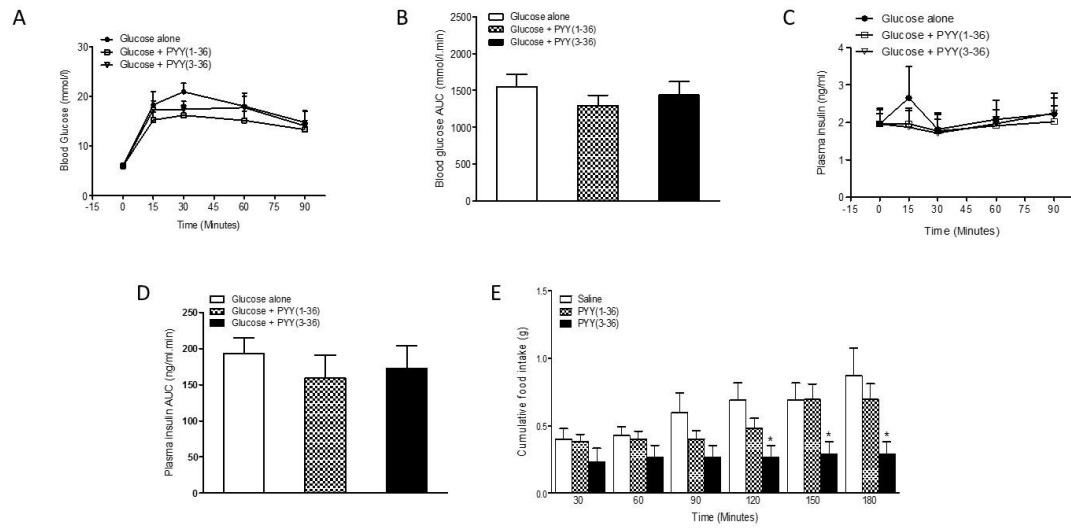


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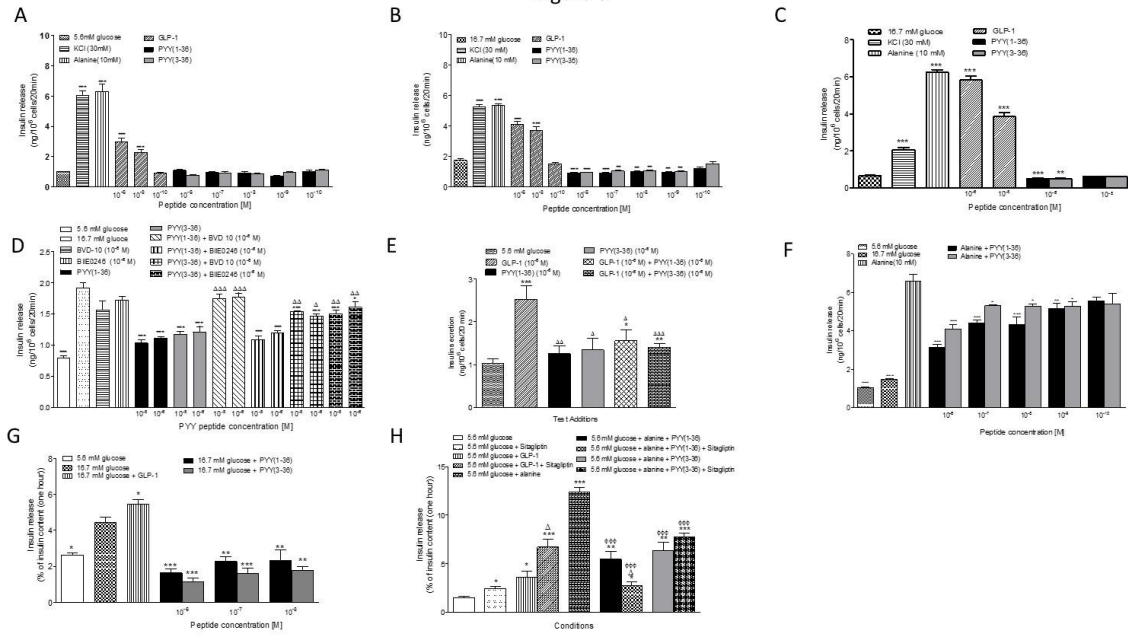


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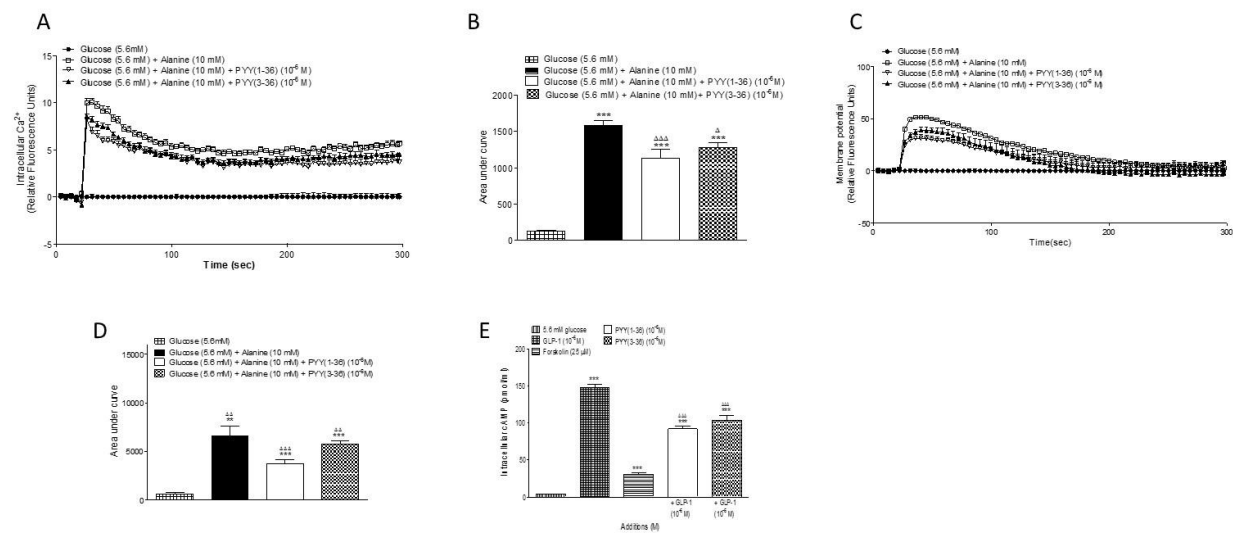


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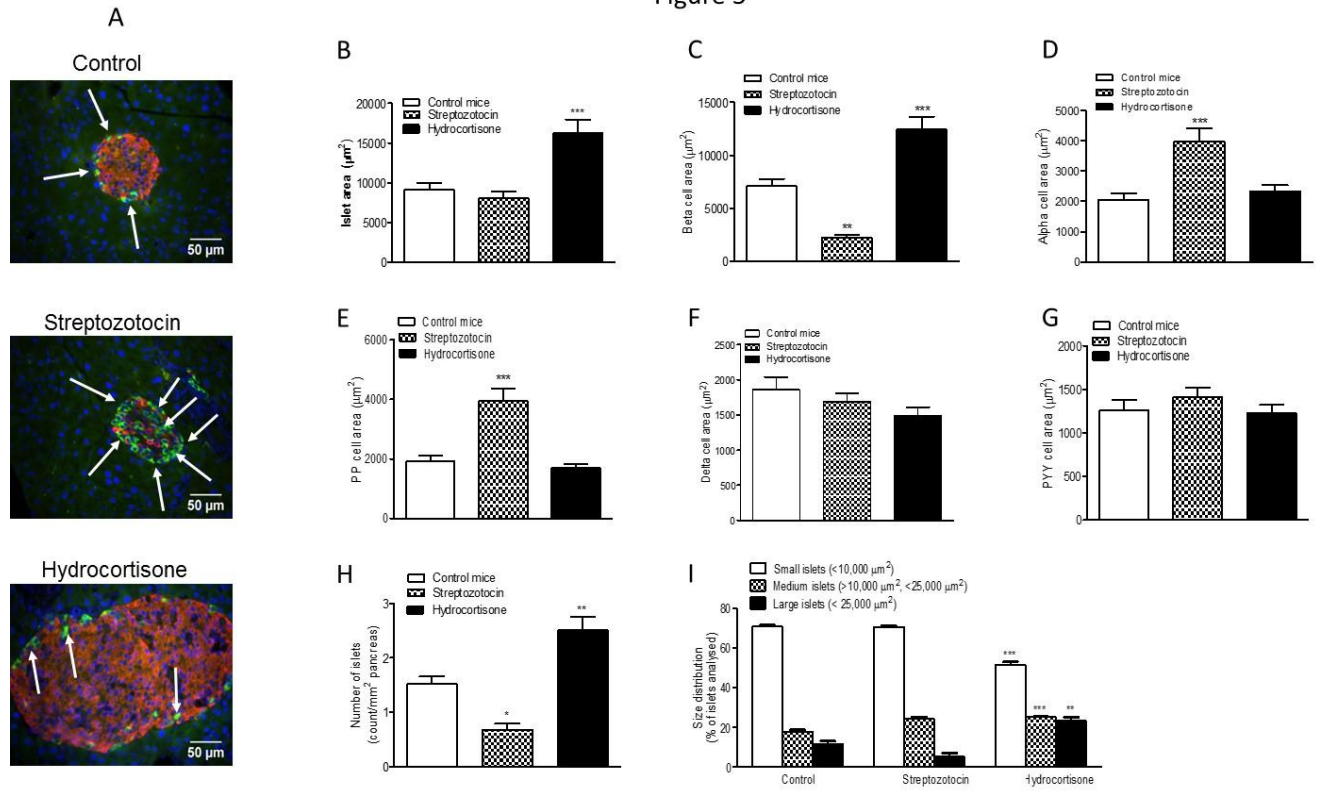


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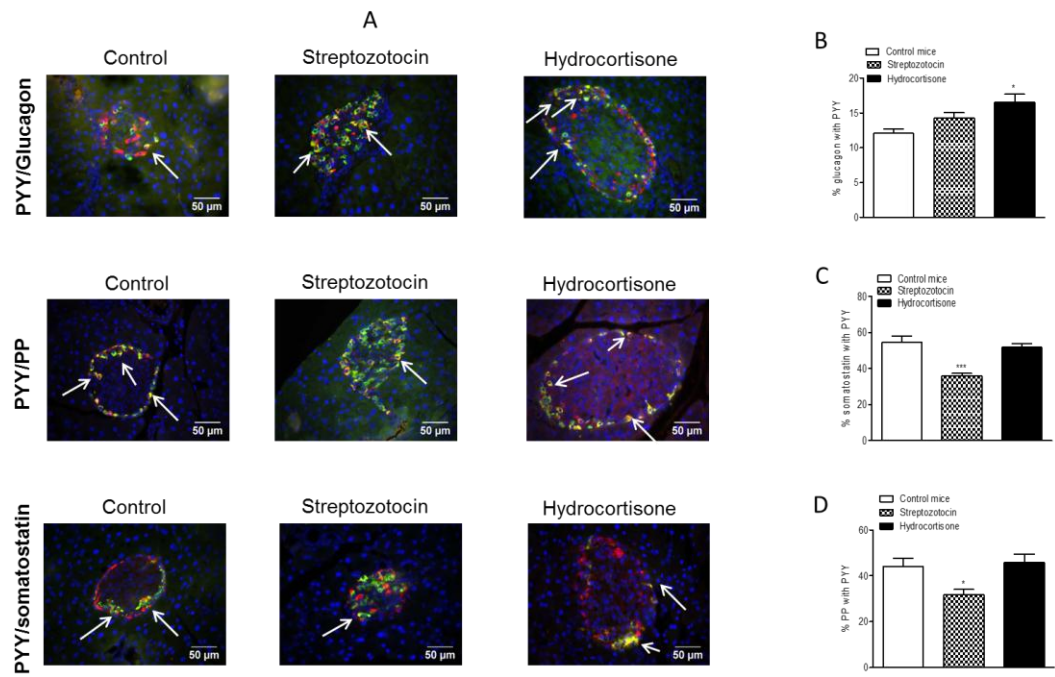


Figure 7

Figure 7

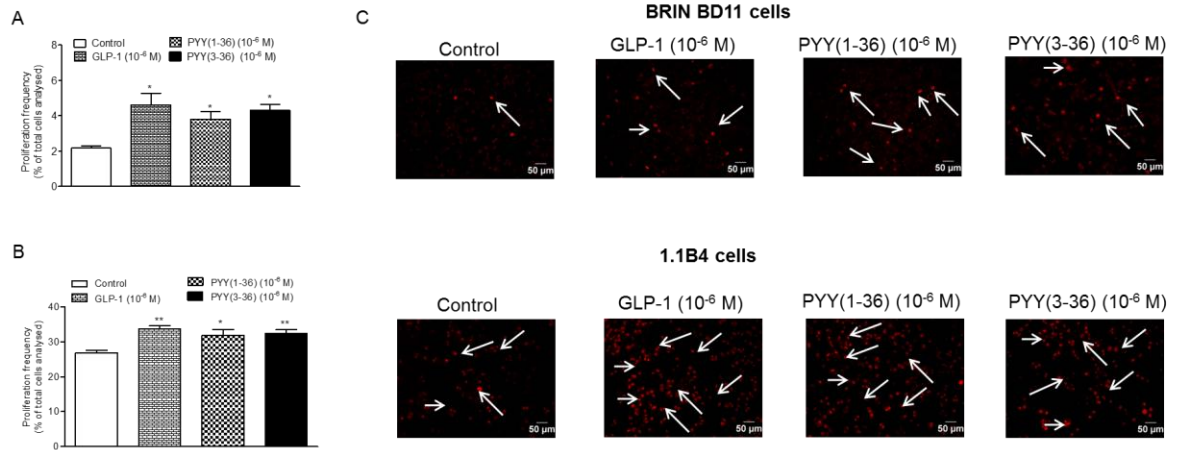
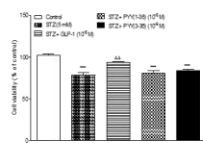
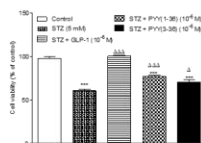


Figure 7

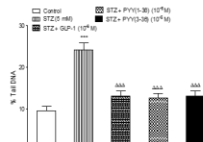
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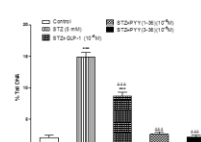
B 1.1B4 cells



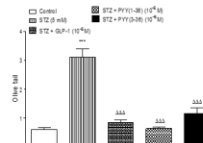
C



D



E



F

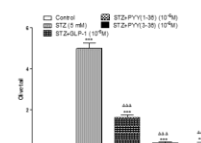


Figure 8

